

Quantitative Real-Time PCR

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Abstract

Unlike classical end-point analysis PCR, real-time PCR provides the data required for quantification of the target nucleic acid. The results can be expressed in absolute terms by reference to external quantified standards or in relative terms compared to another target sequence present within the sample. Absolute quantification requires that the efficiency of the amplification reaction is the same in all samples and in the external quantified standards. Consequently, it is important that the efficiency of the PCR does not vary greatly due to minor differences between samples. Careful optimisation of the PCR conditions is therefore required. The use of probes in quantitative real-time PCR improves its performance and a range of suitable systems is now available. Generally quantitative real-time assays have excellent performance characteristics including a wide dynamic range, high sensitivity and accuracy. This has led to their use in a wide range of applications and two examples are presented. Viral quantification is now an important factor in the control of infection. The problems associated with virus quantification in cytomegalovirus (HCMV) infection are similar to those presented by other viruses. Quantitative PCR is finding an increasing role in the diagnosis of cancer. The assessment of *c-erbB2/Her2/neu* gene duplication is useful in predicting the disease

prognosis in breast cancer. Several different real-time quantitative PCR protocols are available for these applications and have been applied successfully to their respective diseases.

Introduction

The early years of the PCR were not without difficulty. One of the greatest barriers to successful exploitation was the problem of contamination that becomes evident due to the high sensitivity of the method. It was also quickly realised that the term ‘quantitative PCR’ could almost be considered an oxymoron due to the non-linearity of the relationship between amplicon yield and starting copy number. The combined effect of these factors was that even a single amplicon contaminating a reaction mixture could give a false positive result. The practical uses of PCR were therefore restricted and there remained many applications requiring highly sensitive and quantitative nucleic acid detection assays that could not be addressed. This led to the development of fixes that rendered PCR at least semi-quantitative. This was generally achieved by limiting the number of temperature cycles to ensure that reactions containing template in the desired quantitative range did not pass the linear phase of product accumulation. Truly quantitative but relatively inconvenient methods were also developed. These were based on either the competitive target or limiting dilution approaches. These methods are used for RNA and DNA copy number measurement in applications such as viral load estimation and gene expression studies. The widespread use of quantitative PCR was therefore already well established by the time real-time PCR machines were introduced.

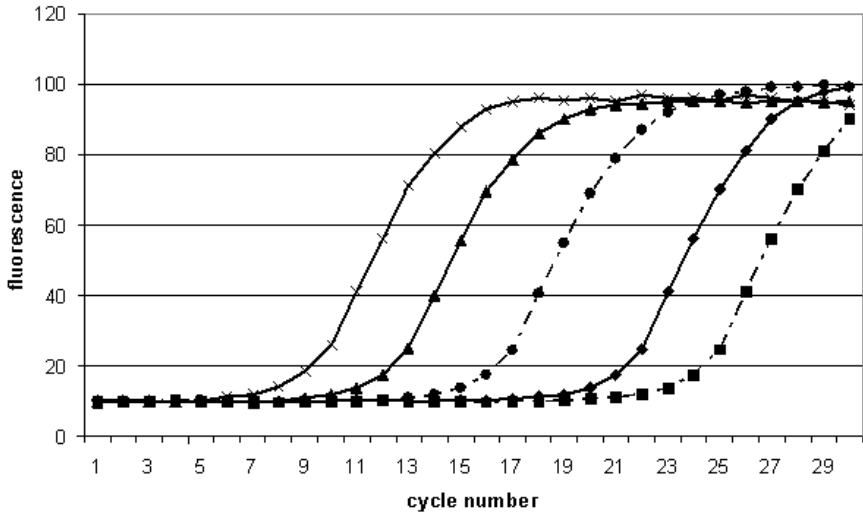
The problem with using PCR quantitatively stems from the variable kinetics of product accumulation after different numbers of cycles. Although the process is essentially exponential during the early cycles this rate cannot be maintained when nanogram/ μ l quantities of product are present. The main reasons for this are the increasing level of amplicon reannealing during the priming step and the overstretching of reaction resources including polymerase, primers and nucleotides as the quantity of DNA to be synthesized during the elongation phase

increases. During this phase product accumulation is approximately linear for a number of cycles. Finally, if cycling is continued beyond the linear phase the quantity of amplicon may reach a plateau when new synthesis of full-length product is only sufficient to replace material lost by the combined effects of hydrolysis at elevated temperatures and exonuclease activity.

There are now two main approaches used to work around the problematic kinetics of PCR in order to obtain accurate quantitative data. The first is competitive quantitative PCR that relies on the assumption that a competitor target added to the PCR reaction will be amplified at the same rate as the authentic target provided that they have identical primer binding sites and broadly similar structures. The input level of target molecules is calculated from the final ratio of the two products. The main disadvantage of this method is that its precision depends upon the accuracy with which the target to competitor ratio can be determined at the end of the amplification process. Generally, this ratio can be measured with greatest accuracy when it is close to unity. This limits the range of quantitative values that can be determined accurately in a single tube containing a set number of copies of the competitor molecule. Running parallel tubes containing different numbers of copies of the competitor can extend the accurate quantitative range of competitive PCR.

The second main approach to quantification by PCR relies on the use of real-time machines. The rationale behind the method depends upon the experimental evidence and theoretical prediction that product accumulation within PCR reactions generally remains exponential for many cycles. While the concentration of product remains very low the effects of sample variation on the amplification efficiencies of different reactions remain small. For example, any low-level polymerase inhibitors introduced with the samples are less likely to prevent the completion of the full-length second strands when the polymerase is present in large excess. The exponential phase only ends when either the level of product reaches a level of several ng/ μ l or when the reaction is 'poisoned' due to the accumulation of primer artefacts. This means that during the exponential phase there is a linear relationship between \log_{10} starting template copies and the number of

A



B

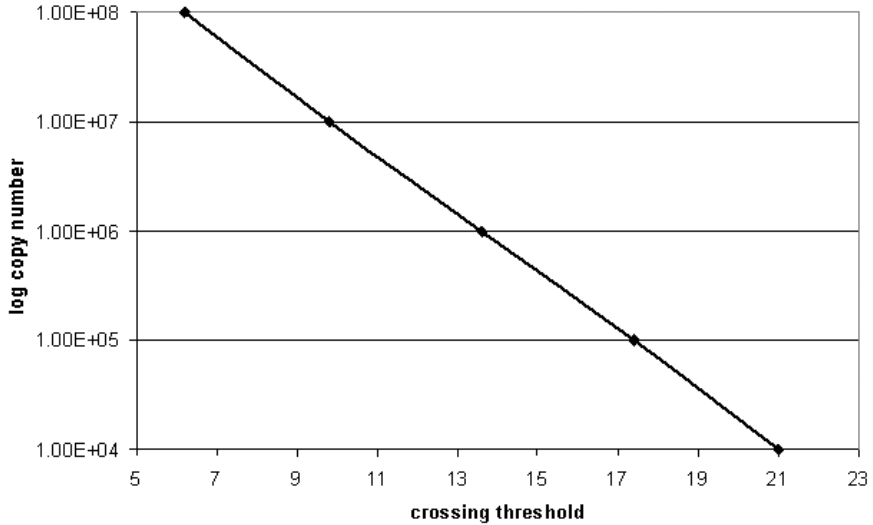


Figure 1. Quantitative real-time PCR. Panel A represents amplification profiles for a series of \log_{10} dilutions of a standard target at 1×10^4 (squares) to 1×10^8 (crosses) copies per reaction. Panel B shows the straight line relationship between the copy number and crossing threshold.

amplification cycles required for the product to reach a set or threshold concentration (Figure 1). In theory the threshold should be set at the lowest reliably measurable level, since the accuracy of quantification should be highest when the measurements are made at the earliest possible stage within the exponential phase. This general approach has proved to be very accurate and reliable.

The measurement of product threshold levels in real-time quantitative PCR is done by analysis of the plots of fluorescence against cycle number. The simplest approach is to set a threshold fluorescence value and then determine the number of cycles required for the fluorescence to reach this level. The threshold level can be set automatically by the software which determines the standard deviation of the points forming the baseline. The threshold is then set at, for example, three standard deviations above the baseline mean. An alternative method is to determine the second derivative maximum for each curve describing fluorescence versus cycle and use this as the threshold value (LightCycler® Online Resource Site at <http://www.lightcycler-online.com/>). This is the point at which the rate of change of fluorescence is fastest and usually occurs at the end of the exponential phase. The fluorescent signal is relatively high at this point and can therefore be measured relatively accurately. The second derivative maximum method looks promising in theory but generally in practice the accuracy of quantitative PCR is greater when the data are analysed by the baseline method.

Standards and Controls for Quantitative Real-Time PCR

Inhibition Controls

PCR inhibitors introduced into the reaction, usually with the sample, may present a barrier to successful quantification. Although it can be assumed that low levels of inhibition will have no effect on the efficiency of PCR before the crossing threshold has been reached, this will not be the case at higher inhibitor levels. In some situations it may be adequate to identify any samples containing inhibitors from their

‘flat’ curves or from the complete absence of an amplification product. However, it may be necessary to include experimental controls that indicate the presence of inhibitory materials (Figure 2). The choice is between internal and external control templates. External controls are simply PCR targets that are amplified under the same conditions as the test material. These are spiked into parallel reaction tubes that are otherwise identical to the experimental tubes. Although it would be possible to use the test sequence as the external control this approach is not advised as it may cause or complicate the investigation of instances of PCR contamination. A better solution is to use a modified version of the target sequence that has been modified by deletion, insertion or substitution within the inter-primer region so that it can be identified specifically in real-time (Kearns *et al.*, 2001; Kearns *et al.*, 2002). The advantage of using an external control is that it cannot affect the conditions within the experimental reaction vessel. The main disadvantage is that an additional reaction is needed for each sample. Internal controls follow the same design as external controls but are spiked into the experimental reaction mixture. Following amplification

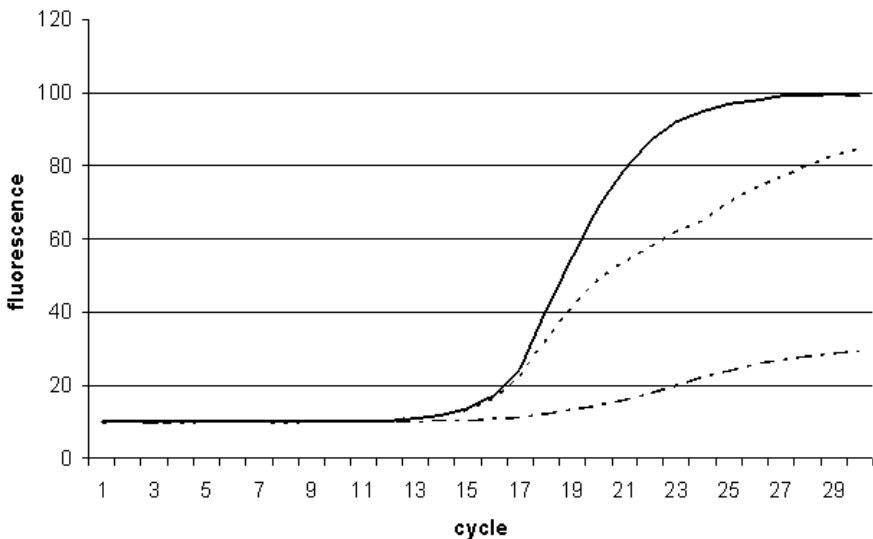


Figure 2. Effect of inhibitors on amplification profiles. The solid line shows an uninhibited reaction in comparison with a reaction that contains some inhibitory material (dotted) and one that is more strongly inhibited (dots and dashes).

it is necessary to distinguish between the two products and this can be achieved by using detection probes that can be distinguished in the real-time instrument. It is also essential to ensure that the internal control is added at the lowest concentration that is consistently detectable. This reduces the possibility of interference between the two reactions that might decrease the efficiency of experimental target amplification. One consequence of the use of low levels of the internal inhibition control is that, due to competition, it will not be amplified to the detection threshold if the target sequence is present at high concentrations. However, under such circumstances the inhibition control is redundant.

External Quantification Controls

Conventionally external quantification controls have precisely the same sequence as the target to be quantified. However, it may be advantageous to introduce a minor sequence modification to allow amplicons derived from the control sequence to be distinguished from the target amplicons in cases of suspected contamination. If the modified base is placed at the probe site it should be possible to identify the quantification control in real-time. Sequence variations at other sites must be detected by alternative means. Stocks of reliably quantified standards of the desired sequence can be prepared using the protocol outlined below.

The accuracy of quantification by real-time PCR depends on the quality of the standard curve. The copy number range of the standards should always encompass the range expected in the experimental samples. The maximum increment between quantitative standards should be \log_{10} and a minimum of four points should be included in each curve. The question arises of whether it is necessary to prepare a separate standard curve for each machine run. Whilst this is undoubtedly the best practice and should give the greatest quantitative accuracy since it eliminates inter-run variation it may not always be a practical solution. An intermediate solution would be to prepare standard curves periodically but to include one quantified standard in each run to check for any drift.

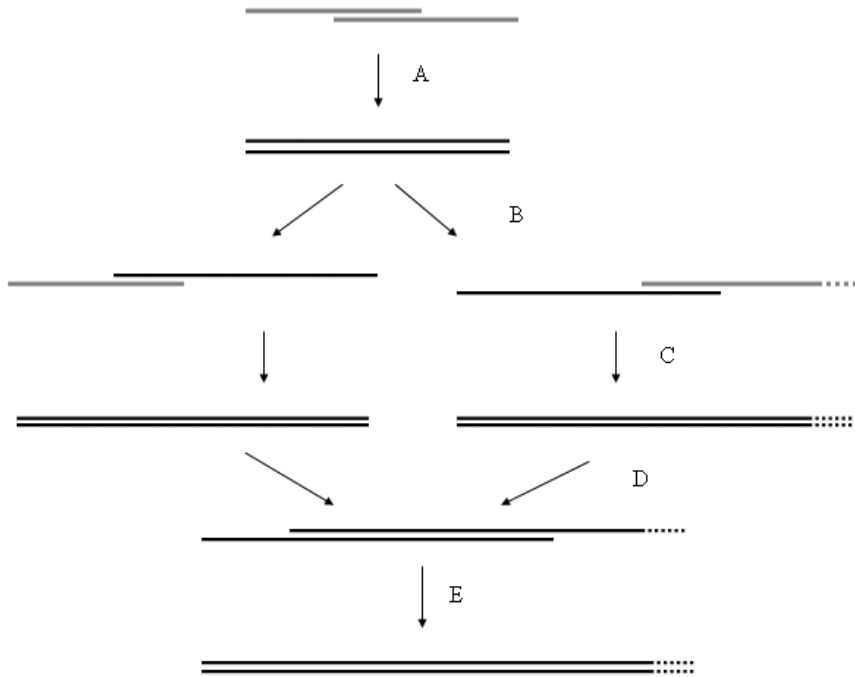


Figure 3. Synthesis of standards for use in real-time quantification assays. Synthetic oligonucleotides are shown in grey and products in black. In step A, primers with complementary 3' ends are mixed and subjected to PCR cycling in the presence of thermostable polymerase and deoxynucleotide substrates. This reaction produces a double stranded product that is mixed with two further primers (step B) complementary to the new 3' ends. If RNA standards are required a T7 RNA polymerase promoter sequence is added to the 5' end of one primer (dotted line). In steps C to E PCR cycling is again used to produce a double stranded product. Step C is the addition of the sequences carried by the second set of primers to the complementary strands. Step D is the annealing of the products of step C which are converted into the double stranded form in step E. Amplification of the final product then continues under the direction of the second primer pair. It may be possible to mix all of the primers into a single mixture but this may not give a good yield in all cases and purification of the first product may be required.

A Simple Approach for the Preparation of Inhibition and Quantification Controls for Real-Time Quantitative PCR

The first step is to design the control template *in silico*. For inhibition controls this is done simply by modifying the target sequence at its probe binding site. It is best to rearrange the relevant bases so that the level of homology between the old and new probe-binding sites is <50%. The new sequence should be identical to the original sequence in terms of the primers and amplification conditions required but should not be detected by the original probe. For quantification controls a single base substitution is sufficient to allow the control to be identified. Check that no stable secondary structures have been introduced into the sequence using suitable computer software (*e.g.* OLIGO 6; Molecular Biology Insights, Cascade, Colorado).

If the inhibition control is to be approximately 140 bases or less, it is simplest to produce it by PCR synthesis starting with extended primers (up to 70-80 bases) that have 20 base complementary ends. Longer controls can be built up in steps of up to 120 bases using additional extended primers (Figure 3). Following the final stage of amplification the control template should be gel purified (*e.g.* QIAquick Gel Extraction kit; Qiagen, Crawley, West Sussex) and then carefully quantified. Alternatively, the control sequence may be cloned into a plasmid vector (*e.g.* using the TOPO kit, Invitrogen, Paisley, Scotland), grown in *Escherichia coli* cells and then purified using a suitable mini-prep kit. It is advisable to verify the sequence of the cloned insert at this point. The simplest and in many cases the most reliable method of quantification is to compare the intensity of a stained band with a commercial quantitative gel standard (*e.g.* 100bp Ladder; New England Biolabs, Hitchin, Herts). This method is most accurate when the bands compared have approximately equal intensities.

Stocks of standard materials should be stored frozen at the highest available concentration in Tris EDTA (TE) buffer. Dilutions of the standards should be made in a solution of carrier nucleic acid (*e.g.* 5µg/ml of sheared salmon sperm DNA dissolved in deionised water) to prevent the loss of significant numbers of copies adhering to the

walls of the storage vessel. It is also important to minimise the loss of the standard DNA by avoiding repeated freeze/thaw cycles.

If RNA standards are required a T7 RNA polymerase promoter sequence should be included at the 5' end of one of the primers (Figure 3). The PCR product may then be used as the substrate in a reaction containing T7 RNA polymerase and the four nucleotides. Residual template can be removed from the reaction using RNase-free DNase prior to quantification of the RNA. RNA standards should be handled and stored very carefully to avoid introducing RNase. One option for reducing the potential problem of nuclease damage is to add an RNase inhibitor such as RNasin (Promega, Madison, WI) to all stocks.

Probes

Quantitative real-time PCR is possible when double stranded DNA binding dyes (*e.g.* SYBR Green I) are used to report the accumulation of the amplicon. However, the accuracy and reliability of this method depends upon the quality of the reaction chemistry to a greater extent than when a sequence specific probe detection method is used. The reason for this is that in the dye system PCR artefacts can be interpreted as copies of the target molecule. When oligonucleotide probe reporters are used this problem does not arise.

Although not recommended for quantitative PCR, the DNA binding dye methods have the potential advantage that the level of signal generated is relatively high and this results in high signal:noise ratios. High signal to noise ratios give smooth plots of fluorescence against cycle number and allow crossing thresholds or maximum delta fluorescence (ΔF) values to be determined with great precision. Since the signal:noise ratio of the fluorescent signal is important in determining the accuracy of quantification by real-time PCR it must also be an important consideration in choosing the probe system to be employed. It is important to select a system that generates strong signals (see Chapter 3) since this will minimise the variation between replicate measurements.

Dynamic Range, Sensitivity and Reliability of Real-Time Quantification

Dynamic Range

A characteristic feature of real-time PCR is that it offers accurate quantification over a very wide range of concentrations. At the top end of the range the limit is set by the need to establish a base-line level of fluorescence before product accumulation is detected, since after this point it is not possible to determine an accurate crossing threshold. In practice this means that it is possible to measure up to approximately 10^{10} copies of template. The bottom end of the quantification scale is set by the sensitivity of the PCR. This may be increased by pre-amplification of the target sequence using external primers (*i.e.* nested PCR). Care must be taken to ensure that the pre-amplification reaction does not progress beyond the exponential phase. This can be done by using a limited number of first-round cycles (Brechtbuehl *et al.*, 2001). Although nested reactions can detect single copies of the target sequence it is unrealistic to expect accurate quantification at very low copy numbers due to sampling errors.

Linearity of Real-Time Quantitative PCR

Plots of \log_{10} initial template copies against the number of cycles required to reach the crossing threshold (or second derivative maximum) in real-time PCR are expected to yield a straight line. This relationship may break down at low copy numbers resulting in either an increase or a decrease in the number of cycles required to give a \log_{10} increase in copy number. Apparent increases in the efficiency of the reaction may occur at low copy number when the intercalating method of product detection is used. This is due to detection of the accumulated double stranded primer artefacts after many PCR cycles. Real decreases in PCR efficiency actually occur as primer artefacts reach high levels due to the depletion of reaction resources. This decrease becomes evident when sequence-specific detection methods are used.

Accuracy

There are two main sources of error in quantification assays that rely upon real-time PCR. The first is the accuracy with which the reaction threshold can be measured. This is largely determined by the level of noise in the baseline signal and depends on the signal to noise ratio. Different real-time machines and detection chemistries vary in this parameter. Probably the most important factor is that the signal transduction chemistry chosen gives a strong signal. The second major source of error is likely to be variations in the sample that have small but cumulatively significant effects on the amplification efficiency. A single-cycle difference between measurements of the crossing threshold will result in a two-fold under- or over-estimation of the initial copy number. Errors of this magnitude are difficult to eliminate completely and in some systems may be even higher. In contrast, sample pipetting errors have a relatively small effect on the overall accuracy of real-time quantification. Although these should be minimised they are unlikely to contribute significantly to the overall accuracy of the real-time quantification.

The errors in real-time PCR quantifications can be minimised and measured by performing replicate analyses. In general, as noted for pipetting errors, variations during sample preparation are relatively small compared with those introduced in the real-time PCRs. Consequently, although ideally many replicates of multiple samples should be tested, in practice it is most advantageous to test a single sample preparation in many real-time tubes. As a minimum, testing in triplicate is recommended.

Reaction Efficiency

The efficiency of PCR reactions can be estimated from the slope of the crossing threshold against copy number line. Most PCR reactions involving short products with normal levels of secondary structure are close to 100% efficient so that a \log_{10} increase in the concentration of the target amplicons requires approximately 3.3 cycles. Efficiency is equal to $E=10^{-1/\text{slope}}$ so that 100% efficiency gives a value of 2

(Bernard and Wittwer, 2002). Higher apparent levels of efficiency are difficult to explain from PCR theory and may indicate that the standards have not been prepared accurately or that other errors have occurred. Poor optimisation of the PCR reaction conditions can result in low efficiency. For example, annealing at too high a temperature or denaturing at a temperature that is too low will both reduce the PCR efficiency. Often the low efficiency of a PCR reaction results from secondary structures within the template. An example of this would be any structure at one of the primer binding sites that inhibits annealing. Low efficiency PCRs (*i.e.* $E=1.5$) can be used for quantification without compromising accuracy. Long PCR products are also associated with poor reaction efficiency. The main reason for this, at least during the early rounds of the PCR, is misincorporation of bases resulting in chain termination which occurs at a higher frequency per round of amplification for longer products. The major disadvantage of the use of an inefficient PCR is that the dynamic range is unlikely to extend to the lower copy numbers.

Inhibitor Effects

PCR inhibitors are substances that either reduce the activity of the polymerase directly or that compete with the target template limiting the availability of polymerase or other resources. At the start of the PCR the polymerase, primers and nucleotides are all present in great excess and consequently any inhibitors may have zero net effect on the efficiency of the reaction. The effect of any inhibition will then increase roughly in proportion with the quantity of DNA to be synthesised. Accurate quantification is not possible for samples that contain inhibitors that reduce the efficiency of amplification significantly before the reaction threshold has been crossed. It is therefore important to use extraction methods that eliminate all non-trivial sources of PCR inhibition.

Absolute or Relative Quantification?

For absolute quantification the number of target template copies present within a unit quantity of sample is determined. This depends upon the

reliability of target nucleic acid extraction and may therefore present a problem for cellular material that is difficult to lyse. It also depends, crucially, on the quality and uniformity of the sample. So, for example, sputum samples examined for the presence of pathogenic organisms are likely to present serious problems for absolute quantification due to their non-uniformity. One effective solution to these difficulties is to perform relative quantifications in which the measured quantity of target sequence is expressed as a ratio with the quantity of a reference sequence as the denominator. In the example of tissue samples a suitable reference might be a human gene. Levels of induced/repressed mRNAs can be expressed most usefully as comparisons with quantities of housekeeping gene transcripts or rRNA subunits. The estimation of the levels of gene duplication in cancer cells is another application where ratios are far more meaningful than absolute quantification values (Bernard and Wittwer, 2002).

Examples of the Use of Quantitative Real-Time PCR

The Measurement of Human Cytomegalovirus (HCMV) Levels in Infected Human Serum

Primary infection with HCMV is usually asymptomatic although occasionally it may lead to a mononucleosis-like syndrome with prolonged fever, and a mild hepatitis. Although the immune system suppresses the virus in healthy individuals, infection results in lifelong persistence of the viral genome. Congenital infection is more significant and results in serious complications in a proportion of cases. Recurrent disease may occur if the immune system is suppressed and this is associated with morbidity and mortality especially in bone marrow or solid-organ transplant recipients and in patients with AIDS. Early diagnosis of active infection is important in high-risk patients since antiviral (ganciclovir) treatment is available and pre-emptive therapy reduces the incidence and severity of disease. Consequently, patients at risk are usually monitored by methods such as pp65 antigen detection or viral DNA PCR (Boeckh and Boivin, 1998). Quantitative PCR for HCMV has been greatly simplified by the use of real-time machines

and is becoming the method of choice for early detection of rises in the HCMV titre or for monitoring the progress of antiviral therapy (Kearns *et al.*, 2001).

Several real-time quantitative PCR assays for HCMV have been reported in the literature (Gault *et al.*, 2001; Guiver *et al.*, 2001; Kearns *et al.*, 2001; Cortez *et al.*, 2003). Most of these methods provide absolute quantification of number of copies of viral DNA per millilitre of serum. However, in one case (Sanchez and Storch, 2002) the quantity of the viral DNA was normalised to the quantity of human DNA present. The sequences targeted in these studies varied although the conserved gene encoding glycoprotein B was a popular choice. In general, the length of the PCR products was rather longer than might be preferred for real-time PCR. It has been reported that serum HCMV is highly fragmented (Boom *et al.*, 2002) and consequently the use of long PCR targets might be expected to impose an additional limit on the sensitivity of these assays. The quantified external controls used in these assays were produced by cloning the PCR product into a suitable plasmid vector. These control plasmids are generally linearised and then quantified. In one study (Kearns *et al.*, 2001) the cloned sequence was modified at two bases within the probe binding site. This has the advantage that the PCR product of plasmid amplification can be distinguished from viral product by the probe melting temperature and thus contamination of samples with plasmid DNA may be recognised. Internal controls that are amplified by the HCMV specific primers but with a different internal sequence were used in the protocol described by Cortez (Cortez *et al.*, 2003). The internal control, which is added to all mixes as an inhibition check, is distinguished from the HCMV product by using different hybridisation probe pairs. Protocols that use either hybridisation (double check) or hydrolysis (TaqMan) probes on either a block based (ABI 7700) or air (LightCycler) have been described. The relative merits of these instruments are discussed elsewhere (Chapter 2).

Assessment of *c-erbB2/Her2/neu* Gene Duplication in Breast Cancer Tissue

An increase in the number of copies of cellular oncogenes caused by DNA amplification often leads to deregulated protein expression and is associated with neoplastic transformation. It has been reported (Slamon *et al.*, 1987) that *c-erbB2/Her2/neu* gene amplification from 2 to >20 fold is found in 30% of human breast tumours. The degree of amplification is correlated with a poor prognosis for a range of tumours including those of breast tissue (Slamon *et al.*, 1987; Press *et al.*, 1997). Investigation of the significance of gene duplication requires that the analytic methods are reliable, accurate and sensitive. Accuracy is required because the extent of duplication is relatively limited. High sensitivity is needed because tissue biopsies are often of limited weight and it may be necessary to obtain material by microdissection (Glockner *et al.*, 2000; Lehmann *et al.*, 2000).

The development of quantitative real-time PCR methods has greatly facilitated the measurement of gene duplication in breast cancer (Glockner *et al.*, 2000; Lehmann *et al.*, 2000; Bernard and Wittwer, 2002; Gunnarsson *et al.*, 2003; Konigshoff *et al.*, 2003). The published assays generally use either the LightCycler or the ABI 7700 combined with appropriate signal transduction chemistries. In contrast with the methods for quantification of HCMV, which usually provide an absolute measure of the number of copies of the virus, methods for measuring *c-erbB2/Her2/neu* gene duplication usually provide a ratio. Several different denominator genes have been used including those for albumin, β -globin, the amyloid precursor and deoxycytidine kinase. These are all single copy genes in normal cells. One group (Glockner *et al.*, 2000; Lehmann *et al.*, 2000) employed two control genes. This approach has the advantage that it is more likely to reveal cases of control gene duplication that might cause underestimation of the experimental gene copy number. All of the methods cited above measure relative gene copy numbers by analysis of the experimental and control targets within a single tube. If all of the PCR reactions in the multiplex have identical levels of efficiency, the amplification curves of the different targets will have the same crossing threshold, providing no gene duplication has occurred. In tumours with gene duplication

of one of the targets the crossing threshold for the duplicated gene shifts to a lower value. This method is able to detect gene duplication with great sensitivity. The magnitude of the duplication can then be estimated accurately from the crossing threshold shift and the efficiency of the PCR.

One group (Lyon *et al.*, 2001; Millson *et al.*, 2003) have devised an interesting method that uses the capabilities of a real-time PCR instrument (the LightCycler) to perform quantitative competitive PCR. The competitor was a modified version of the genomic *c-erbB2/Her2/neu* target that differed by a single base substitution in the probe binding site. The target and competitor could be effectively distinguished by the 8°C difference between the melting curve maxima. Plots of the ratio of competitor to target melting peak area against competitor copy number for a fixed amount of test genomic DNA gave a good fit to a third-order polynomial curve. Over a limited range of ratios the linear fit was also reasonably accurate. For practical detection of *c-erbB2/Her2/neu* gene duplication, 50ng aliquots of test DNA were tested against 15,000 copies of competitor sequence. This single point was used to find the copy number from the known third-order polynomial curve. The gene dose estimations by this method for twenty tumour samples correlated well with values determined by quantitative real-time PCR.

Conclusions

Real-time quantitative PCR is now well established as a method for estimating quantities of specific nucleic acid sequences. One major advantage is that it may be applied to samples containing levels of the target sequence that are several \log_{10} units lower than can be detected using hybridisation based methods. Another is that this sensitivity contributes to a dynamic range that can be $>8 \log_{10}$ units in a single assay. In comparison with alternative methods such as competitive PCR real-time quantification is simpler and more accurate. Relative quantification is particularly well suited to the new real-time instruments since the control and experimental genes may be tested under identical conditions in the same tube.

The drawbacks of quantitative real-time PCR are few and minor, with the exception of the capital outlay needed for the initial purchase of a real-time instrument. However, the cost of the instrumentation is declining rapidly in real terms and this is likely to continue in parallel with increases in performance, reliability and ease of use. The cost per test of quantitative real-time PCR (excluding capital cost) is similar to that of qualitative real-time PCR which can now be lower overall than that of standard PCR with an agarose gel end-point analysis.

Future Developments

Real-time PCR instrument development continues to deliver machines that are more rapid, accurate and sensitive than their forerunners. In addition, the associated control and analysis software is becoming more intuitive and capable. In parallel with the advances in the instrumentation, improved chemistries are delivering greater analytical sensitivity and improved specificity. These improvements are resulting in an increase in the accuracy of quantitative PCR based on relative crossing threshold measurements.

Arrays are now being used for quantitative assays, particularly for measurement of the levels of individual mRNAs within complex mixtures. Clearly, such parallel systems are extremely attractive in the appropriate circumstances. The main limitations of arrays are their relatively low sensitivities and dynamic ranges. In future, improved instrumentation and systems for amplification on the chip may become more viable and allow arrays to encroach further into the domain of quantitative PCR. However, for the present real-time PCR generally provides higher quality data than can be obtained from arrays and should remain an important complementary method due to the reliability of the quantitative data generated.

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